CLAIMS

1. The use of nucleotide sequences chosen from those comprising a sequence coding for a protein of the trimethylamine N-oxide reductase (TMAO reductase) system in bacteria, or a fragment of this sequence such as a probe or a primer of about 15 to about 25 nucleotides, or a sequence derived by addition, suppression, and/or substitution of one or more nucleotides of this sequence coding for a protein of the TMAO reductase system or of a fragment of the latter, the said fragment and the said derived sequence being capable of hybridizing with the said sequence coding for a protein of the TMAO reductase system, for implementing a method of detecting the presence of all bacteria involved in the process of degradation of the flesh of aquatic animals, in a host that can be a carrier of the said bacteria.

2. Use according to Claim 1, characterized in that the sequence coding for a protein of the TMAO reductase system is chosen from the sequences coding for TMAO reductase in bacteria, further designated TorA or DorA protein, or coding for a type *c* cytochrome, further designated TorC or DorC protein.

3. Use according to Claim 1 or Claim 2, characterized in that the nucleotide sequences coding for a protein of the TMAO reductase system are chosen from those of the following bacteria:

– marine bacteria, such as those of the genus *Shewanella*, *Photobacterium* or *Vibrio*, the said sequences being chosen in particular from the following:

* the sequence coding for the TorA protein of *Shewanella massilia* shown in Figure 1,

* the sequence coding for the TorA protein of *Shewanella putrefaciens* shown in Figure 1,

* the sequence coding for the TorA protein of *Shewanella c* shown in Figure 2,

* the partial sequence coding for the TorA protein of *Photobacterium phosphoreum* shown in Figure 3,

* the sequence coding for the TorC protein of *Shewanella massilia* shown in Figure 14,

– bacteria obtained from brackish water, such as those of the genus *Rhodobacter*, or *Roseobacter*, the said sequences being chosen in particular from the following:

* the sequence coding for the DorA protein of *Rhodobacter sphaeroides* shown in Figure 4,

* the sequence coding for the DorA protein of *Rhodobacter capsulatus* shown in Figure 4,

* the sequence coding for the DorC protein of *Rhodobacter sphaeroides* shown in Figure 14,

– enterobacteria, such as those of the genus *Escherichia*, or *Salmonella*, the said sequences being chosen in particular from the following:

* the sequence coding for the TorA protein of *Escherichia coli* shown in Figure 4,

* the partial sequence coding for the TorA protein of *Salmonella typhimurium* shown in Figure 5,

* the sequence coding for the TorC protein of *Escherichia coli* shown in Figure 14.

4. Use according to any one of the Claims 1 to 3, characterized in that the nucleotide sequences are used in the form of pairs of primers chosen from any one of the following three groups of primers:

(1) the group of primers "DDN" comprising:

◆ the following compositions of nucleotide sequences "DDN+":

– DDN1+ : *5′* CGG vGA yTA CTC bAC hGG TGC *3′* : mixture of 54 nucleotide sequences,

– DDN5+ : *5′* ATy GAT GCG ATy CTC GAA CC *3′* : mixture of 4 nucleotide sequences,

◆ the following compositions of nucleotide sequences "DDN-":

– DDN2- : *5′* CGT Amw sGT CGA kAT CGT TrC GCT C *3′* : mixture of 32 nucleotide sequences,

– DDN3- : *5′* GAC TCA CAy Awy TGy GAG TG *3′* : mixture of 16 nucleotide sequences,

– DDN4- : *5′* TGr CCd CGr kCG TTA AAG AC *3′* : mixture of 24 nucleotide sequences,

– DDN5- : *5'* CCv GGT TCG AGr ATC GCA TC *3'* : mixture of 6 nucleotide sequences,

(2) the group of primers "BN" comprising:

◆ the following compositions of nucleotide sequences "BN+":

– BN1+ : *5'* C bGA yAT CsT rCT GCC *3'* : mixture of 16 nucleotide sequences,

– BN3+ : *5'* GGm GAy TAy TCb ACm GGy GC *3'* : mixture of 96 nucleotide sequences,

– BN6+ : *5'* Twy GAr CGy AAC GAy mTC GA *3'* : mixture of 64 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BN-":

– BN2- : *5'* GG vyC rTA CCA bsC vCC TTC *3'* : mixture of 216 nucleotide sequences,

– BN4- : *5'* ATC Arr CCn swv GGC GTG CC *3'* : mixture of 192 nucleotide sequences,

– BN5- : *5'* GbC ACr TCd GTy TGy GG *3'* : mixture of 72 nucleotide sequences,

(3) the group of primers "BC" comprising:

◆ the following compositions of nucleotide sequences "BC+":

– BC1+ : *5'* ACn CCn GAr AAr TTy GAr GC *3'* : mixture of 256 nucleotide sequences,

– BC2+ : *5'* TGy ATh GAy TGy CAy AAr GG *3'* : mixture of 96 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BC-":

– BC2- : *5'* CCy TTr TGr CAr TCd ATr CA *3'* : mixture of 96 nucleotide sequences,

– BC3- : *5'* TTn GCr TCr AAr TGn GC *3'* : mixture of 128 nucleotide sequences,

in which n = (A,C,G,T), y = (C,T), r = (A,G), h = (A,C,T), d = (G,A,T), m = (A,C), w = (A,T), b = (G,T,C), s = (G,C), v = (G,A,C), and k = (G,T),

the pairs of primers being chosen in such a way that one of the primers of a pair corresponds to one of the aforementioned compositions of nucleotide sequences DDN+, BN+ or BC+, whereas the other primer corresponds respectively to one of the

aforementioned compositions of nucleotide sequences DDN-, BN- or BC-, the said pairs of primers being chosen in particular from any one of the following four pairs:

(a) the pair DDN1+/DDN5-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, of a size of about 820 base pairs (bp), and especially to the amplification of an 821 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 821 bp fragment bounded by the nucleotides located in positions 620 to 1450 of the *tor*A gene of *S. massilia* shown in Figure 4,

(b) the pair BN6+/BN2-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, with a size of about 710 bp, and especially to the amplification of a 727 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 727 bp fragment bounded by the nucleotides located in positions 1657 to 2403 of the *tor*A gene of *S. massilia* shown in Figure 4,

(c) the pair BN6+/BN4-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, with a size of about 360 bp, and especially to the amplification of a 355 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 355 bp fragment bounded by the nucleotides located in positions 1657 to 2023 of the *tor*A gene of *S. massilia* shown in Figure 4,

(d) the pair BC1+/BC2-, leading to amplification of fragments of the gene coding for the TorC protein in bacteria, with a size of about 170 bp, and especially to the amplification of a 197 bp fragment of the gene coding for the TorC protein in bacteria of the genus *Shewanella*, such as the 197 bp fragment coding for the polypeptide fragment bounded by the amino acids located in positions 114 to 179 of the TorC protein of *S. massilia* shown in Figure 14.

5. Use according to one of the Claims 1 to 4, characterized in that the hosts that can be carriers of bacteria involved in the process of degradation of the flesh of aquatic animals, as described in Claim 3, are aquatic organisms, especially marine organisms such as fish and crustacea, and more particularly Atlantic fish such as sole, cod, or fish from the Mediterranean Sea such as surmullet and sea bream, as well as certain animals from fresh or brackish water.

6. Use according to any one of the Claims 1 to 5, for implementing a method of detecting the presence of all bacteria involved in the degradation of the flesh of

aquatic animals, within the framework of a method of evaluating the freshness of aquatic animals from which the sample tested was taken, when the said animals are removed from their natural environment.

7. A nucleotide sequence corresponding to one of the following sequences:

- DDN1+ : *5'* CGG vGA yTA CTC bAC hGG TGC *3'*,
- DDN5+ : *5'* ATy GAT GCG ATy CTC GAA CC *3'*,
- DDN2- : *5'* CGT Amw sGT CGA kAT CGT TrC GCT C *3'*,
- DDN3- : *5'* GAC TCA CAy Awy TGy GAG TG *3'*,
- DDN4- : *5'* TGr CCd CGr kCG TTA AAG AC *3'*,
- DDN5- : *5'* CCv GGT TCG AGr ATC GCA TC *3'*,
- BN1+ : *5'* C bGA yAT CsT rCT GCC *3'*,
- BN3+ : *5'* GGm GAy TAy TCb ACm GGy GC *3'*,
- BN6+ : *5'* Twy GAr CGy AAC GAy mTC GA *3'*,
- BN2- : *5'* GG vyC rTA CCA bsC vCC TTC *3'*,
- BN4- : *5'* ATC Arr CCn swv GGC GTG CC *3'*,
- BN5- : *5'* GbC ACr TCd GTy TGy GG *3'*,
- BC1+ : *5'* ACn CCn GAr AAr TTy GAr GC *3'*,
- BC2+ : *5'* TGy ATh GAy TGy CAy AAr GG *3'*,
- BC2- : *5'* CCy TTr TGr CAr TCd ATr CA *3'*,
- BC3- : *5'* TTn GCr TCr AAr TGn GC *3'*,

in which n = (A,C,G,T), y = (C,T), r = (A,G), h = (A,C,T), d = (G,A,T), m = (A,C), w = (A,T), b = (G,T,C), s = (G,C), v = (G,A,C), and k = (G,T).

8. A composition of nucleotide sequences mixed together, corresponding to one of the following compositions:

◆ the following compositions of nucleotide sequences "DDN+":

– DDN1+ : *5'* CGG vGA yTA CTC bAC hGG TGC *3'* : mixture of 54 nucleotide sequences,

– DDN5+ : *5'* ATy GAT GCG ATy CTC GAA CC *3'* : mixture of 4 nucleotide sequences,

◆ the following compositions of nucleotide sequences "DDN-":

– DDN2- : *5'* CGT Amw sGT CGA kAT CGT TrC GCT C *3'* : mixture of 32 nucleotide sequences,

– DDN3- : *5′* GAC TCA CAy Awy TGy GAG TG *3′* : mixture of 16 nucleotide sequences,

– DDN4- : *5′* TGr CCd CGr kCG TTA AAG AC *3′* : mixture of 24 nucleotide sequences,

– DDN5- : *5′* CCv GGT TCG AGr ATC GCA TC *3′* : mixture of 6 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BN+":

– BN1+ : *5′* C bGA yAT CsT rCT GCC *3′* : mixture of 16 nucleotide sequences,

– BN3+ : *5′* GGm GAy TAy TCb ACm GGy GC *3′* : mixture of 96 nucleotide sequences,

– BN6+ : *5′* Twy GAr CGy AAC GAy mTC GA *3′* : mixture of 64 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BN-":

– BN2- : *5′* GG vyC rTA CCA bsC vCC TTC *3′* : mixture of 216 nucleotide sequences,

– BN4- : *5′* ATC Arr CCn swv GGC GTG CC *3′* : mixture of 192 nucleotide sequences,

– BN5- : *5′* GbC ACr TCd GTy TGy GG *3′* : mixture of 72 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BC+":

– BC1+ : *5′* ACn CCn GAr AAr TTy GAr GC *3′* : mixture of 256 nucleotide sequences,

– BC2+ : *5′* TGy ATh GAy TGy CAy AAr GG *3′* : mixture of 96 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BC-":

– BC2- : *5′* CCy TTr TGr CAr TCd ATr CA *3′* : mixture of 96 nucleotide sequences,

– BC3- : *5′* TTn GCr TCr AAr TGn GC *3′* : mixture of 128 nucleotide sequences,

in which n = (A,C,G,T), y = (C,T), r = (A,G), h = (A,C,T), d = (G,A,T), m = (A,C), w = (A,T), b = (G,T,C), s = (G,C), v = (G,A,C), and k = (G,T).

9. A pair of primers, characterized in that it is chosen from one of the following groups of primers:

(1) the group of primers "DDN" comprising:

- the following compositions of nucleotide sequences "DDN+":
  - DDN1+ : *5'* CGG vGA yTA CTC bAC hGG TGC *3'* : mixture of 54 nucleotide sequences,
  - DDN5+ : *5'* ATy GAT GCG ATy CTC GAA CC *3'* : mixture of 4 nucleotide sequences,
- the following compositions of nucleotide sequences "DDN-":
  - DDN2- : *5'* CGT Amw sGT CGA kAT CGT TrC GCT C *3'* : mixture of 32 nucleotide sequences,
  - DDN3- : *5'* GAC TCA CAy Awy TGy GAG TG *3'* : mixture of 16 nucleotide sequences,
  - DDN4- : *5'* TGr CCd CGr kCG TTA AAG AC *3'* : mixture of 24 nucleotide sequences,
  - DDN5- : *5'* CCv GGT TCG AGr ATC GCA TC *3'* : mixture of 6 nucleotide sequences,

(2) the group of primers "BN" comprising:

- the following compositions of nucleotide sequences "BN+":
  - BN1+ : *5'* C bGA yAT CsT rCT GCC *3'* : mixture of 16 nucleotide sequences,
  - BN3+ : *5'* GGm GAy TAy TCb ACm GGy GC *3'* : mixture of 96 nucleotide sequences,
  - BN6+ : *5'* Twy GAr CGy AAC GAy mTC GA *3'* : mixture of 64 nucleotide sequences,
- the following compositions of nucleotide sequences "BN-":
  - BN2- : *5'* GG vyC rTA CCA bsC vCC TTC *3'* : mixture of 216 nucleotide sequences,
  - BN4- : *5'* ATC Arr CCn swv GGC GTG CC *3'* : mixture of 192 nucleotide sequences,
  - BN5- : *5'* GbC ACr TCd GTy TGy GG *3'* : mixture of 72 nucleotide sequences,

(3) the group of primers "BC" comprising:

◆ the following compositions of nucleotide sequences "BC+":

– BC1+ : *5'* ACn CCn GAr AAr TTy GAr GC *3'* : mixture of 256 nucleotide sequences,

– BC2+ : *5'* TGy ATh GAy TGy CAy AAr GG *3'* : mixture of 96 nucleotide sequences,

◆ the following compositions of nucleotide sequences "BC-":

– BC2- : *5'* CCy TTr TGr CAr TCd ATr CA *3'* : mixture of 96 nucleotide sequences,

– BC3- : *5'* TTn GCr TCr AAr TGn GC *3'* : mixture of 128 nucleotide sequences,

in which n = (A,C,G,T), y = (C,T), r = (A,G), h = (A,C,T), d = (G,A,T), m = (A,C), w = (A,T), b = (G,T,C), s = (G,C), v = (G,A,C), and k = (G,T),

the pairs of primers being chosen in such a way that one of the primers of a pair corresponds to one of the aforementioned compositions of nucleotide sequences DDN+, BN+ or BC+, whereas the other primer corresponds respectively to one of the aforementioned compositions of nucleotide sequences DDN-, BN- or BC-, the said pairs of primers being chosen in particular from any one of the following four pairs:

(a) the pair DDN1+/DDN5-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, of a size of about 820 base pairs (bp), and especially to the amplification of an 821 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 821 bp fragment bounded by the nucleotides located in positions 620 to 1450 of the *tor*A gene of *S. massilia* shown in Figure 4,

(b) the pair BN6+/BN2-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, with a size of about 710 bp, and especially to the amplification of a 727 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 727 bp fragment bounded by the nucleotides located in positions 1657 to 2403 of the *tor*A gene of *S. massilia* shown in Figure 4,

(c) the pair BN6+/BN4-, leading to amplification of fragments of the gene coding for the TorA protein in bacteria, with a size of about 360 bp, and especially to the amplification of a 355 bp fragment of the gene coding for the TorA protein in bacteria of the genus *Shewanella*, such as the 355 bp fragment bounded by the nucleotides located in positions 1657 to 2023 of the *tor*A gene of *S. massilia* shown in Figure 4,

(d) the pair BC1+/BC2-, leading to amplification of fragments of the gene coding for the TorC protein in bacteria, with a size of about 170 bp, and especially to the amplification of a 197 bp fragment of the gene coding for the TorC protein in bacteria of the genus *Shewanella*, such as the 197 bp fragment coding for the polypeptide fragment bounded by the amino acids located in positions 114 to 179 of the TorC protein of *S. massilia* shown in Figure 14.

10. A method of detecting all bacteria involved in the degradation of the flesh of aquatic animals in a host that can be a carrier of the said bacteria, the said method being effected starting from a biological sample taken from the said host, the said biological sample corresponding in particular to a subcutaneous fragment of flesh of the aquatic animal in question, and being characterized in that it comprises a step of hybridization of at least one nucleotide sequence as defined in any one of the Claims 1 to 9, with fragments of genes coding for a protein of the TMAO-reductase system of bacteria involved in the degradation of the flesh of aquatic animals that can be present in the biological sample taken from the said host, followed by a step of detection, in particular by electrophoresis, of the possible presence, in the said sample, of genes coding for a protein of the TMAO-reductase system, or of fragments of these genes, of which the number of copies has been amplified if necessary.

11. A method of detection according to Claim 10, characterized in that it comprises the following steps:

– treatment of a biological sample taken from this host in order to extract the total DNA from this host and render the genome of these bacteria accessible to the nucleotide sequences or primers defined according to any one of the Claims 1 to 9, the said treatment being effected in particular using a technique of rapid DNA extraction based on fixation of the nucleic acids on silica beads,

– amplification of the number of copies of genes coding for the proteins of the TMAO-reductase system of bacteria involved in the degradation of the flesh of aquatic animals, or of fragments of these genes, which can be present in this sample, with the aid of the aforementioned nucleotide sequences or primers hybridizing with the aforementioned genes or fragments of genes,

– detection of the possible presence of an amplified number of copies of genes coding for a protein of the TMAO-reductase system of the aforementioned bacteria, or

of fragments of these genes, and hence of the presence of the said bacteria in the biological sample investigated.

12. A method of detection according to Claim 11, characterized in that amplification of the number of genes coding for the TMAO-reductase comprises the following steps:

– predenaturation of the total double-stranded DNA of the host to single-stranded DNA, preferably in a buffer consisting of 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, of the 4 constituent deoxynucleotides of the DNAs (dCTP, dATP, dGTP, dTTP) at a concentration of 100 μM each, and of the primer pairs as defined in Claims 1 to 9, by heating between about 90°C and about 100°C, advantageously at 94°C, for about 1.5 min,

– actual amplification, by adding DNA polymerase, for example Taq polymerase, to the medium obtained in the preceding step,

♦ heating at about 94°C for about 30 seconds, which corresponds to the actual denaturation step,

♦ then heating between about 35°C to about 60°C, and especially at about 45°C or 55°C, for about 30 seconds, which corresponds to the step of hybridization of the primers with the genes coding for the proteins of the TMAO-reductase system of bacteria, or of the fragments of these genes, which can be present in the biological sample investigated,

♦ and finally, heating at 72°C, for about 45 seconds, which corresponds to the step of extension of the primers hybridized in the preceding step, towards one another, thus producing complementary nucleotide sequences of fragments of genes coding for the proteins of the TMAO-reductase system of bacteria, these last-mentioned sequences being bounded by the nucleotides that hybridize with the aforementioned primers,

– repetition of the preceding amplification step between about 15 and about 35 times, advantageously about 30 times.

13. A kit for implementing a method of detection according to one of the Claims 10 to 12, characterized in that it comprises:

– one or more nucleotide sequences or primers defined in one of the Claims 1 to 9,

– a DNA polymerase,

– a reaction medium advantageously consisting of 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin, the 4 constituent deoxynucleotides of the DNAs (dCTP, dATP, dGTP, dTTP) at a concentration of 100 μM each.

14. A nucleotide sequence comprising:

– the sequence shown in Figure 2 of the torA gene coding for the TorA protein of the marine bacterium *Shewanella c*,

* or any sequence derived from the aforementioned sequence by degeneration of the genetic code, and coding for the TorA protein of *Shewanella c*, the peptide sequence of which is shown in Figure 6,

* or any sequence derived from the aforementioned nucleotide sequence, especially by substitution, suppression or addition of one or more nucleotides, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned nucleotide sequence shown in Figure 2,

* or any fragment of the aforementioned nucleotide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 15 nucleotides,

– the partial sequence shown in Figure 3 of the gene coding for the TorA protein of the marine bacterium *Photobacterium phosphoreum*,

* or any sequence derived from the aforementioned sequence by degeneration of the genetic code, and coding for the TorA protein of *Photobacterium phosphoreum* the peptide sequence of which is shown in Figure 7,

* or any sequence derived from the aforementioned nucleotide sequence, especially by substitution, suppression or addition of one or more nucleotides, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned nucleotide sequence shown in Figure 3,

* or any fragment of the aforementioned nucleotide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 15 nucleotides,

– the partial sequence shown in Figure 5 of the gene coding for the TorA protein of the marine bacterium *Salmonella typhimurium*,

* or any sequence derived from the aforementioned sequence by degeneration of the genetic code, and coding for the TorA protein of *Salmonella typhimurium* the peptide sequence of which is shown in Figure 8,

* or any sequence derived from the aforementioned nucleotide sequence, especially by substitution, suppression or addition of one or more nucleotides, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned nucleotide sequence shown in Figure 5,

* or any fragment of the aforementioned nucleotide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 15 nucleotides.

15. A peptide sequence encoded by a nucleotide sequence according to Claim 14, and comprising:

– the amino acid sequence shown in Figure 6 of the TorA protein of *Shewanella c*,

* or a sequence derived from the aforementioned peptide sequence, especially by substitution, suppression or addition of one or more amino acids, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned peptide sequence shown in Figure 6,

* or a fragment of the aforementioned peptide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 5 amino acids,

– the partial amino acid sequence shown in Figure 7 of the TorA protein of *Photobacterium phosphoreum*,

* or a sequence derived from the aforementioned peptide sequence, especially by substitution, suppression or addition of one or more amino acids, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned peptide sequence shown in Figure 7,

* or a fragment of the aforementioned peptide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 5 amino acids,

– the partial amino acid sequence shown in Figure 8 of the TorA protein of *Salmonella typhimurium*,

* or a sequence derived from the aforementioned peptide sequence, especially by substitution, suppression or addition of one or more amino acids, the said derived sequence preferably having a homology of about 35% to 100% with the aforementioned peptide sequence shown in Figure 8,

* or a fragment of the aforementioned peptide sequence, or of a sequence derived from the latter as defined above, the said fragment preferably being constituted of at least about 5 amino acids.